Investigation of poly(styrene-divinylbenzene-vinylsulfonic acid) as retentive and electroosmotic flow generating phase in open-tubular electrochromatography

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ABSTRACT

In this work, a new sulfonated polystyrene based porous layer was synthesized on the wall of a capillary by a single step in situ polymerization process. To obtain a capillary suited for electrochromatography, vinylsulfonic acid (VSA) was, next to divinylbenzene (DVB), copolymerized to induce charges for the electroosmotic flow (EOF) generation. The VSA ratio in the monomer mixture and the polymerization time were optimized while the chromatographic characteristics of the obtained open tubular columns were investigated in electrochromatography. To allow unambiguous study of only chromatographic processes, evaluations were performed with a mixture of sufficiently retained and electrophoretically neutral parabens. Comparison of SEM pictures and chromatograms revealed that the polymerization time had a great influence on the polymer layer morphology and on the chromatographic performance. An increase in the VSA ratio, led to an increase in the mobile phase velocity but simultaneously lowered paraben retention. The novel optimized stationary phase could generate a stable and significant electro-osmotic flow (EOF) of 1.1 mm/s over a wide pH range which could be produced in a reproducible manner. Minimal plate heights of 10 µm, equivalent to the capillary internal diameter, were obtained. The open-tubular character of this optimized porous layer column allowed successful analyses at elevated temperature, resulting in a maximum efficiency of 85,500 plates for a 75 cm capillary and linear velocities up to 1.4 mm/s. Finally, a thermal gradient was successfully applied, leading to artificial sharpened peaks with a peak capacity of 55 in a 20 min time span.

1. Introduction

Capillary electrochromatography (CEC) has regularly been appraised as the solution in the quest for a generic, highly efficient and miniaturized separation method. This separation technique combines the high selectivity of HPLC with the high separation efficiency of capillary electrophoresis. In this technique, the separation of the solutes will be the result of partitioning between the stationary phase and the mobile phase which can be superimposed by electrophoresis in case of charged solutes [1-5]. The high efficiencies are achieved by the plug-like flow profile of electrodriven techniques, which causes less band broadening compared to pressure driven techniques. Furthermore, the length of the capillary is not limited by the pressure drop and therefore longer and more efficient columns can be used [6-11].

A great variety of column types have already been applied in CEC. These can be mainly divided in three formats, whereas the vast majority of research has been done on the packed bed format, followed closely by the monolithics. However, comparatively little research has been performed on the open-tubular format.

The achievable high efficiency in CEC has extensively been demonstrated for packed capillaries [12-15]. However, packed-column CEC is hindered by some significant practical problems such as the fabrication of the frits and by the difficulty to pack micrometer sized particles in the narrow-bore capillaries in a homogeneous way. The frits retain the packing material in the capillary and are predominantly made out of sintered silica material [16-18]. Therefore, the frits are often dissimilar from the packed bed and cause inhomogeneities, resulting in a poorer performance and the formation of gaseous bubbles [19]. Moreover, the classical packing materials consist out of silica particles, which are functionalized. Therefore, it can often be difficult to separate basic compounds due to the presence of these silanol groups, needed to generate an adequate EOF. Furthermore, to achieve reproducible analyses in electrodriven methods, the same amount of silanol groups should be deprotonated for each analysis. This is achieved by rinsing the capillary with a highly basic solution, followed by water and the mobile phase. However, the silica particles will deteriorate at an pH lower than 2 or higher than 8. Hence, this reconditioning cannot be applied and therefore packed CEC is less reproducible.

The development and performance of monolithic columns in CEC has also been investigated

quite extensively. These are prepared by the in-situ polymerization of organic or inorganic precursors in the presence of a porogen and are covalently linked to the silanized wall. These monoliths can be divided in two categories: organic monoliths and inorganic monoliths and in principle eliminate most of the practical problems experienced with packed capillaries. There is no need for retaining frits, narrow columns (20-100 μm) can be prepared and no gaseous bubbles will be formed during analysis (hence there is no need for pressurization during analysis) [20-24].

The organic porous monoliths in CEC are typically based on acrylamide, methacrylate and styrene polymers, whereas research has mostly been focused on methacrylate based monoliths [22,25,26]. However, polystyrene monoliths are broadly successfully applied for highly efficient separations of biomolecules in HPLC. These monoliths are chemically stable under a wide pH range and the morphology can be adjusted by the copolymerization of other monomers and the selection of the proper porogenic solvent [27-29]. Despite their widespread use in HPLC, the research on the use of polystyrene monoliths (and stationary phase in general) in CEC is still fairly limited. Horvath et al. investigated the potential of bimodal CEC monoliths for the analysis of peptides and proteins [30]. Jin et al. and Xiong et al. applied a one-step polymerization with methacrylic acid as EOF generating comonomer to obtain a negatively charged polystyrene monolith [31,32]. They obtained efficient separations on a 20 cm column of aromatic compounds and biomolecules with respectively 28,000 and 18,000 plates. However, the efficiency of highly retained small and uncharged organic compounds (k> 1.5) was not investigated. Furthermore, methacrylic acid only allows for an adequate EOF at pH 7 or higher. To achieve generation of EOF at lower pH's Huang et al. changed the charge bearing monomer to the strong acids vinyl sulfonic acid (VSA) and vinyl benzene sulfonic acid (VBSA). Separation of parabens and acidic compounds at a wide pH range (3-8) were obtained and after optimizing the polymerization conditions, a maximum efficiency of 8.800 (VSA) and 16.400 plates (VBSA) for 20 cm columns (effective length) was observed [33,34]. The porosity of the monoliths can be controlled by changing the porogen or the composition of the monomer mixture. Various approaches have been described to characterize the monolith porosity [Journal of Chromatography A, 1325 (2014) 115– 120].

Nevertheless, monolithic capillaries are often less efficient compared to packed particle columns and the column to column reproducibility is often poor. The lower encountered efficiencies can be associated with wall effects caused by the shrinkage of the polymer network during the curing step of the monolith. On the other hand, it has been demonstrated that open-tubular chromatography can be a viable and possible alternative for the packed and monolithic format. These capillaries also depict some advantages compared to the traditional applied packed bed capillaries including the lack of need for frit formation, the relative easiness of preparation and stationary phase modification. More importantly, open-tubular CEC offers significant practical benefits such as the possibility to cut off clogged sections of the capillary, easier rinsing and the facile hyphenation with ESI-MS. Moreover, open tubular capillaries should demonstrate a somewhat better reproducibility compared to monolithic capillaries. Unlike the packed and monolithic format, the performance of open- tubular capillaries is directly linked to the internal diameter of the capillary. Small internal diameters are required to facilitate efficient solute diffusion into the stationary phase [35]. More narrow capillaries provide higher efficiency and a higher concentration of an on column loaded sample [36]. Last but not least, the joule heating in open-tubular columns will be non-existing or limited due to the small internal diameters (5-20 µm). The latter might allow the effective use of temperature gradients in CEC and could solve the two-decade long problem of gradient analysis in CEC without major modification of the commercial instrumentation. However, the loading capacity and retention of many OT-LC (and CEC) capillaries is limited due to the high phase ratio ($\beta = V_m/V_s$, whereby V_m and V_s correspond to the volume of mobile and stationary phase, respectively). A porous layer of polymer, attached to wall, will increase the surface area and therefore enhance the loadability of the stationary phase. Karger et al. developed a strategy to form a porous open-tubular crosslinked polystyrene layer, attached to the wall. The layer was prepared by filling a pretreated capillary (to functionalize the wall) with a mixture of the monomers and ethanol as solvent. The polymerization will thereby preferentially take place at the non polar wall instead of in the polar solvent, resulting in the formation of a porous polymer layer at the capillary wall with a vacant center. The proportion of monomer mixture and solvent was critical to achieve a layer at the wall instead of a monolith. The high performance of these capillaries in HPLC was

attested by several proteomic analyses resulting in peak capacities of 400 and whole protein analyses [37-39]. Subsequently, the same group produced an open-tubular HILIC mode capillary by the polymerization of divinylbenzene and vinylbenzylchloride, followed by modification of the polymer wall with ethylenediamine as functional group [40].

Regardless of reports of high efficient analyses of biomolecules obtained by open-tubular polystyrene capillaries in nano-LC, there are very few reports on the use of open-tubular polystyrene based capillaries in CEC (to the best of our knowledge). Huang et al. applied a styrenic stationary phase in 20 µm ID capillaries, modified with quaternary ammonium groups as charge bearing moiety and dodecyl chains as retention sites [41]. Separation of four proteins with a counter-directional electro-osmotic flow (due to the presence of the basic ammonium groups) was obtained in this way. However, the potential of polystyrene based stationary phases is often described through the separations of large biomolecules, while a lower performance is obtained for the separation of small organic molecules. Therefore, an intriguing challenge presents itself in the manufacturing of open-tubular highly performant styrenic capillaries with an acidic EOF generating moiety incorporated. The emphasis in this work was set on the preparation of such an open-tubular capillary by a single step polymerization. VSA was added as pH independent EOF generating component to a monomer mixture of styrene and divinylbenzene. To best of the author's knowledge, it is the first time that VSA was used as comonomer in a styrene-based open-tubular CEC column. Therefore the fundamental performance of this type of column is evaluated in detail only with small, retained, organic and neutral molecules. This at it allows disconnection of the chromatographic performance from electrophoretic phenomena in an unambiguous way. The influence of changes in the polymerization time, monomer composition and effect of elevated analysis temperatures are evaluated to optimize the synthesis and analytical conditions.

2. Experimental

2.1 Reagents and materials

Fused silica capillaries (10 µm ID) were purchased from CMscientific (Silsden, United Kingdom). Milli-Q water was prepared in house by purification and deionization of tap water in a Milli-Q

plus water instrument from Millipore (Bedford, New Hampshire, USA). Acetic acid, acetonitrile, toluene, dichloromethane, ethanol (EtOH) and ammonium acetate of HPLC quality originated from Biosolve (Valkenswaard, Netherlands). Styrene, divinylbenzene, vinylsulfonic acid, trimethoxy silyl propyl methacrylate, azobisisobutyronitrile (AIBN) and all test compounds were obtained from Sigma-Aldrich (Bornem, Belgium). Stock solutions of all compounds were prepared at 10.000 µg/ml in water or ACN, depending on their solubility, and diluted to 125 µg/ml (in the same solvent composition as the mobile phase) prior to analysis.

2.2 Preparation of PLOT Column

The capillaries were prepared as described by Yue et al. with the adaptation that vinyl sulfonic acid is added to the polymerization mixture [37]. Fused-silica capillaries with 10 µm I.D. and a length of 1.5 meter were rinsed for 3 h with 1 M NaOH at 25 bar, washed with water and MeOH and finally dried under N_2 for 1 h to remove residue water and methanol. The 10 µm ID pretreated capillary was filled with a freshly prepared solution of 15% 3-(trimethoxysilyl) propyl methacrylate (v/v) in toluene. The capillary was sealed at both ends and placed in the oven at 120°C for 10 hours. Subsequently the capillary was washed with acetonitrile and dried with nitrogen at 25 bar. Inhibitors present in styrene and divinylbenzene were removed by flushing the liquids through an basic alumina column. The monomer mixture, containing styrene, divinylbenzene and vinyl sulfonic acid, was dissolved in ethanol (40/60 v/v). AIBN (25 mg) was added as initiator to 10 ml of the polymerization mixture. The solution was degassed by ultrasonication for 15 min and subsequently introduced in the capillary. Both ends were sealed and the capillary was placed in the oven at 75°C during 15 -120 min. The column was rinsed with acetonitrile during 30 min to elute un-bounded polymer and subsequently conditioned with the mobile phase at 25 bars during 1 h. A 25 cm part was cut from both capillary ends resulting in a capillary with a total length of 1 meter. Prior to analysis, the open-tubular poly (styrene-dinvinylbenzene-vinnylsulfonic acid) (PSDVB-VSA) capillaries were cut at desired lengths (33.5 - 82.5 cm).

2.3 Chromatographic conditions for CEC

The 7100 CE system (Agilent Technologies, Waldbronn, Germany) used in this work includes an air-cooled thermostat, auto sampler, diode array detector and a built-in system to pressurize vials. The mobile phase was filtered with syringe filters (Grace, 0.45 µm PVDF filters). Injections were performed electrokinetically or hydrodynamically. An alignment interface for standard capillaries of 25 µm ID (with an optical slit of 25 µm) was used. A stock solution of ammonium acetate was prepared by adding acetic acid until a solution with desired pH was obtained. The mobile phase was prepared by adding the required volume of the stock solution to ACN. Consequently, the mobile phase was degassed in an ultrasonic bath for 10 min prior to analysis. Analysis temperature was always maintained isothermal at 25°C, 40°C or 60C. Furthermore, a temperature gradient was applied by increasing the set air temperature. The gradient was kept isothermal for 1 min at 25°C, subsequently the temperature was raised to 60°C with a 2°C/min rate, and kept at this temperature during 12.5 minutes. Detection was performed at 214 and 254 nm and all obtained data was processed with Chemstation B.04.03 software. New columns were conditioned by increasing the voltage to 30 kV in 120 min in a stepwise way..

If not specified otherwise, all shown analyses were obtained at 25°C with a mobile phase consisting of 25 mM ammonium acetate mixed with acetonitrile to a 70/30 ratio and run at an operating voltage of 30 kV. The effective capillary length was 25 cm with a total length of 33.5 cm.

3 Results and Discussion

Ideally, open-tubular columns applied in liquid chromatography should have an internal diameter of 1-2 µm to obtain comparative and even higher efficiencies as in gas chromatography [35]. However, this work was still performed on 10 µm capillaries due to practical considerations (on-column detection, easy clogging during film preparation). The preparation of the polymeric film was reminiscent of PLOT column based work by Karger et al. with some important adaptations [37]. To obtain a sufficient EOF in CEC, an ionizable moiety should be present in the stationary phase. Therefore, a charge bearing monomer should be copolymerized in the polystyrene-divinylbenzene polymer. As the altered polymerization

conditions of this novel approach inevitably influence the morphology and the physical characteristics of the porous polymer layer, these aspects needed to be investigated and optimized. The initial polymerization mixture consisted of a monomer mixture of styrene, divinylbenzene and vinyl sulfonic acid (S/DVB/VSA 1/1/1 v/v) dissolved in ethanol (40/60 v/v). As the initially employed long polymerization time of 16 hours and more resulted in clogged capillaries, this was reduced by more than one order of magnitude to allow the generation of open-tubular capillaries. The polymerization process of the porous layer on the inner lining of the capillary was assessed by scanning electron microscoping (Figure 1A-C) and the corresponding electrochromatographic performance was investigated by the separation of thiourea (as EOF marker) and four parabens, as depicted in Figure 2. The choice of parabens as representative test mixture relies on their electrophoretical neutrality at all applied pH's (3-8) to allow unambiguous occurrence of only chromatographic processes (and not electrophoresis) in all experiments. The small solutes were also selected (and not bio-macromolecules) as they partition between the two phases and are thereby effectively absorbed in the polymeric matrix. The latter allows more fundamental chromatographic insight but also leads to peak broadening due to the slower mass transfer and suboptimal performance at room temperature, as was observed in packed column HPLC before [J. Chromatogr. A 1217 (2010) 3217–3222]. During polymerization the polymer forms spherical shapes, covering the entire wall. The hydrophilic solvent (EtOH) in the center of the capillary tube forces the polymerization to start at the hydrophobically derivatized wall, as the polystyrene polymer is also hydrophobic from nature. As polymerization starts at several points in the capillary next to each other, this results after a time in the formation of a thin porous layer of polymer. Note that this process is analogous to the OTLC column manufactured by Karger et al with the exception that the VSA was thereby absent. However, after 15 minutes (Figure 1A), the growth of the spherical polymer globes is yet not sufficient to cover the entire wall with a relatively uniform layer. In this early stage, the polymer particulates have radii ranging from 40 to 250 nm. The absence of uniformity and the broad size distribution are also reflected in the analysis of thiourea and the parabens on the resulting column (Figure 2A). Although the compounds are distinctively separated, the peak shape is poor and the efficiency is neglectable due to the extensive tailing of the retained

components. Clearly, this can be explained by the discontinuous stationary phase and the broad size distribution.

After 30 minutes, the globular polymer nodules increase in thickness and are linked together, covering the entire wall, as illustrated in Figure 1B. The polymer particulates have now grown to a thickness between 400 and 600 nm. Each nodule is surrounded and bordered by other polymer globes and hence, a wall covering layer of porous polymer is formed. The higher uniformity of the stationary phase layer is reflected in the chromatograms in Figure 2B. Improved peak shapes and increased retention are thereby observed. For example, the retention factor of butylparaben of the 15 min and the 30 min polymerized capillary increased from 1.23 to 1.78, respectively. However, more retained compounds ($k \geqslant 2$) are still tailing, resulting in a maximum efficiency of 4000 plates for butylparaben. On the other hand, a longer polymerization time (1 hour and more) resulted in a thicker layer of maximum 950 nm, but with a broad size distribution (smallest polymer globes are 300 nm), as some polymer globes are limited in their growth by sterical hindrance (Figure 1C). Therefore, there is slightly more retention at long polymerization time but the broader size distribution lowers somewhat the efficiency compared to the optimum polymerization time of 30 minutes and in addition more peak tailing is observed (Figure 2C).

It is clear that the presence of vinyl sulfonic acid in the polymer did not alter the reversed phase separation mechanism and that also a thin film can be produced by this modified polymerization protocol. The presence of VSA in this work is of high importance as this copolymer is responsible for the generation of the EOF.

The presence of a higher amount of VSA groups in the polymer should generate a higher EOF and expedite the analyses. Therefore, the influence of the VSA concentration in the monomer composition on the chromatographic properties was evaluated. Consequently, a series of four open-tubular capillaries were prepared in which the ratio of S/DVB/VSA was altered in the range of 1/1/0.5 to 1/1/3 while the ratio between monomer mixture and solvent (40/60) was maintained. Clearly, the presence of increasing amounts of VSA as comonomer decreased the hydrophobicity of the crosslinked polystyrene layer while coinciding also with lower retention factors of the parabens (Table 1). For example, the retention factor of butylparaben decreases

from 2 to 1.43 for the S/DVB/VSA capillaries with a ratio of 1/1/0.5 and 1/1/2, respectively. In addition, the EOF velocity increases from 0.70 mm/s to 1.09 mm/s, respectively, as depicted in Figure 3. Furthermore, a favorable effect on efficiency and peak shape of the parabens can be remarked if higher VSA ratio's are applied in the monomer mixture. For example, the efficiency of methylparaben increases from 24118 to 27415 when comparing the capillary with the lowest and the highest applied VSA ratio in the monomer mixture, respectively, while the retention factor (k') for the same compound decreases from 0.25 to 0.18, as depicted in Table 1. These effects can be partially explained by the lower retention factor of the compounds but also by the different morphology of the porous polymer layer. A maximum increase of the VSA ratio by a factor 4 resulted in only a 80 % percent increase of the mobile phase velocity, suggesting that the amount of copolymerized VSA groups was not directly proportionate with the increase in the VSA ratio. Due to the possibilities to perform faster and more efficient analyses, the optimized monomer mixture with a S/DVB/VSA ratio of 1/1/2 was used for further research.

Upon further increasing VSA ratio's (larger than 2.5) the polymerization started in every point across the capillary, resulting in a clogged capillary or in the best case a monolithic capillary rather than an open-tubular capillary. The lower hydrophobicity of this new polymer causes a change in the polymerization mechanism, as the polymerization now occurs across the whole capillary, instead of only at the hydrophobic wall. As a consequence the format then changes from open-tubular to monolithic and the solvent, ethanol, served in this case as a porogen. As the goal of this work was to optimize open-tubular conditions, the monolithic format was not further optimized and the chromatographic characteristics of the latter were not evaluated.

The lowering of the retention and the increasing of the mobile phase velocity with increased VSA ratio's in the monomer mixture demonstrates that the VSA monomer is tightly embedded in the porous polymer layer. Furthermore, the FT-IR spectrum of the polymer (polymerized in bulk) showed a chemical shift at 1069 and 1093, which was not present at the FT-IR spectrums of a PS-DVB polymer. These chemical shifts are corresponding with the presence of sulfonic acid. In addition, Figure 4 demonstrates that a sufficiently high and stable EOF generation was generated at both low and high pH conditions suggesting that the EOF is generated by a strong acid, such as vinyl sulfonic acid, which is deprotonated even at low pH conditions. The residual

small dependency of the pH which is observed, can be related to the influence of the higher ionic strength at the lower pH's, as a consequence of the addition of increasing larger amounts of acid, which is known to lower the EOF. Alternatively, the influence of residual silanol groups on the capillary wall is not excluded as well to explain this phenomenon.
The EOF generation of the optimized capillary was sufficiently high to perform analyses up to well into the C-term of the Van Deemter curve, while an analysis of a capillary with PS/DVB/VSA ratio of 1/1/1 was only possible in the B-term, even with the used relatively short columns (25 cm) and applying the highest voltages (30 kV). The Van Deemter curves, illustrated in figure 5, were constructed by analyses of the test mixture at 10 different voltages ranging between 7.5 and 30 kV. Optimal velocities were measured at 0.6 mm/s with a minimal plate height of 9.8 µm for the poorly retained methylparaben which corresponds with the minimal theoretical plate height for open-tubular columns of 10 µm ID ($N= L/H= L/d_c$). However, the Van Deemter curves of ethylparaben en propylparaben reveals a detrimental effect of retention on the minimum plate heights coinciding with minimums of 14.9 and 28 µm, respectively. These reported plate heights and their accordingly steep increase in the slope of the C-term (corresponding with increasingly slower mass transfers) can be explained by the morphology of the porous polystyrene layer. It should be noted that this is fairly consistent with the systematically poor results obtained with poly(styrene-divinylbenzene) based packed columns when analyzing small molecules in HPLC. It appears that also here the diffusion of the small molecules in PSDVB/VSA material is too slow at room temperature [42]. The material should therefore also further be evaluated for the analysis of large molecules, who will, as is the case with PSDVB, not migrate into the material and therefore offer improved results. Furthermore, the open-tubular character of the capillary induces a slow mass transfer kinetic from the mobile phase to the stationary phase, due to the slow diffusion coefficients of liquids (compared to gas).

To examine the reproducibility, the same analyses were performed on the same column in consecutive days. The RSD% of the retention time varied from 0.52 to 0.93% for the day to day repeatability. More imperatively, the reproducibility of the manufacturing process was also investigated by analyzing the same test mixture on 3 columns, prepared in the same manner but on different days. Figure 6 illustrates the overlay of the analysis of the test mixture on the three

columns and the RSD of the retention times was thereby varying between 1.57 to 2.75%. These results indicate that the manufacturing of the porous polymeric open-tubular capillaries is promising in terms of reproducibility and column stability.

As mentioned before, the internal diameter of open-tubular capillaries should in principle be further restricted due to the limited diffusion kinetics of the solutes in aqueous media (compared to gaseous mobile phases). However, the sensitivity of open-tubular columns in CEC will be limited for UV-on column detectors, compared to packed or monolithic columns (ID's 20 to 100 µm). On the other hand, very narrow capillaries dissipate joule heating, created during the analysis and should improve column efficiency and signal to noise ratios. The used narrow open-tubular capillaries are therefore more suited to perform electrodriven analyses even at higher temperatures. In order to investigate this, isothermal analyses were performed at 25°C, 40°C and 60°C. Note that these temperatures were the environment air(cooling) temperature in the vicinity of the capillary, as the actual temperature in the capillary center cannot be measured during analysis. Figure 7A-C illustrates that a higher set temperature coincides with a higher mobile phase velocity, due to the lower viscosity of the mobile phase. An increase in the temperature from 25°C to 60°C corresponded with an increase in mobile phase velocity from 0.95 mm/s to 1.37 mm/s (+40%). Additionally, a drop in retention in function of temperature could also be noted. A reduction in retention factor for butylparaben from 1.0 to 0.67 (-33%) coincided with a rise in temperature of 35°C. As this drop in retention is comparable or even lower compared to the drop in retention in HPLC at elevated temperatures, this individually proves that no problematic Joule heating phenomena is taking place in the capillary during the analysis [43,44]. However, the effect of the elevated temperatures on the efficiency is more ambiguous. Table 2 reveals beneficial effect on the efficiency of methyl- and ethylparaben at a higher set temperatures (40°C), but simultaneously the efficiency decreases at further elevated temperatures (60°C). These observations are directly related to the shifting shape and position of the Van Deemter curves at different temperatures.

More importantly these results indicate interesting possibilities for the application of temperature gradients instead of the, harder to realize, mobile phase compositional gradients, to increase the eluotropic strength in CEC. Note that the measured set temperature in the

cassette is the actual temperature of the air surrounding the capillary and therefore is not necessarily the actual temperature at the capillary center. The authors assume that there will be a small time delay before the set temperature is reached in the capillary center. The optimal temperature gradient ranged between 25 and 60°C, with a gradient rate of 2°C/min. Figure 7D, illustrates a significant drop in the apparent retention factors of the retained components. A comparison between the (apparent) retention factor of the gradient analysis on one hand and the isothermal run at 25°C and 40°C on the other hand, reveals a drop in retention factor for butylparaben of 30 and 17 percent, respectively. Hence, the gradient expedites the analysis, resulting in a faster analysis times. Nevertheless, the retention of butylparaben at 60° (isothermal) was still lower compared to the gradient. The gradient run induced a peak focusing effect coinciding with lower retention time, resulting in sharper peak shapes and less tailing. To measure this effect quantifiably, the average peak capacity was measured of the isothermal runs and the gradient run as described in literature [45-47]. A peak capacity of 55 was obtained for the gradient run (time interval of 20 min), while the peak capacities of the isothermal runs at 25, 40 and 60°C were 50, 54 and 38, respectively. Hence, the gradient run is the more favorable choice due to the reduced analysis time and the improved peak shape while obtaining slightly better peak capacities. Clearly, the influence of temperature in open-tubular CEC needs further investigation. Note, that the velocity of the mobile phase will change during such a temperature based gradient run, in contrary to pressure driven techniques or mobile phase gradients. This change undoubtedly further effects the overall efficiency of the analyses.

4 Conclusion

This work illustrates the development and application of an open-tubular capillary with a porous polymer layer of poly(styrene-divinylbenzene-vinylsulfonic acid) in capillary electrochromatography. The vinylsulfonic acid generated a stable and sufficient highly EOF over a wide pH range. The polymer layer was optimized in terms of polymerization time and monomer composition to obtain an optimum peak shape and EOF generation. This work revealed that as high as possible VSA ratio's (S/DVB/VSA 1/1/2) are beneficial for the analysis time, efficiency and peak shape of the retained compounds. Furthermore, a polymerization of

30 min, resulting in a layer thickness of 400-600 nm seemed to be the optimum time to achieve efficient separation on 10 µm ID capillaries. These optimized preparation conditions led to an optimum plate height of 10 µm. However, the plate heights increase steeply with retention. Therefore, the beneficial effect of performing analyses at elevated temperature were studied. Isothermal analyses at high temperature (40°C) were favorable in terms of efficiency and peak shape but the application of a temperature gradient expedited the analysis time significantly, while preserving the peak capacity.

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Figure Captions

Figure 1: SEM micrographs of polystyrene based open-tubular layers in 10 µm capillaries after a polymerization time of 15 (Fig. 1A), 30 (Fig. 1B) and 60 (Fig. 1C) min.

Figure 2: Effect of the polymerization time on the chromatographic characteristics. The monomer mixture contained styrene/divinylbenzene/ vinyl sulfonic acid (1/1/1 v/v) in a 40/60 v/v ratio to the solvent (ethanol). Total effective length of the capillary was 50 cm, all other analysis parameters were the same as described in the experimental section. A separation of thiourea (1), methyl-(2), ethyl(3), propyl (4) and butylparaben (5) was obtained.

Figure 3: The effect of the VSA ratio in the monomer mixture on the chromatographic characteristics. Three different ratios of S/DVB/VSA were tested: 1/1/0.5 (A), 1/1/1 (B) and 1/1/2 (C). Analysis parameters were the same as described in the experimental section. Compounds and elution order remained the same as in Figure 2.

Figure 4: The effect of the pH of the buffer on the mobile phase velocity

Figure 5: Constructed Van Deemter curves of methylparaben (squares), ethylparaben (circles), propylparaben (triangles). All data points were calculated from the separation of the parabens on a S/DVB/VSA (1/1/2) open-tubular capillary with the same analysis parameters as described in the experimental section.

Figure 6: Overlay of three separations, run with the same conditions but on three different capillaries, prepared at three different days. Mobile phase was composed of ammonium acetate (25 mM, pH 5.5) and acetonitrile (60/40). All other analysis parameters were the same as described in the experimental section. Compounds and elution order remained the same as in Figure 2.

Figure 7: The influence of set air temperature on the chromatographic characteristics. Two isothermal runs at 25°C(A) 40°C (B) and 60°C (C) and a temperature programmed run (D) were depicted. Mobile phase was composed of ammonium acetate (25 mM, pH 5.5) and acetonitrile (60/40). The effective capillary length was 75 cm. All other analysis parameters were the same as described in the experimental section. Compounds and elution order remained the same as in Figure 2.

Table1

	Efficiency N (plates)			Retention factor (k or k_{app})		
Compound	1/1/0.5	1/1/1	1/1/2	1/1/0.5	1/1/1	1/1/2
methylparaben	24118	26922	27415	0.25	0.22	0.18
ethylparaben	20018	22169	24879	0.40	0.35	0.31
propylparaben	5106	5776	7823	0.80	0.73	0.68
butylparaben	3483	4053	4976	2	1.78	1.43

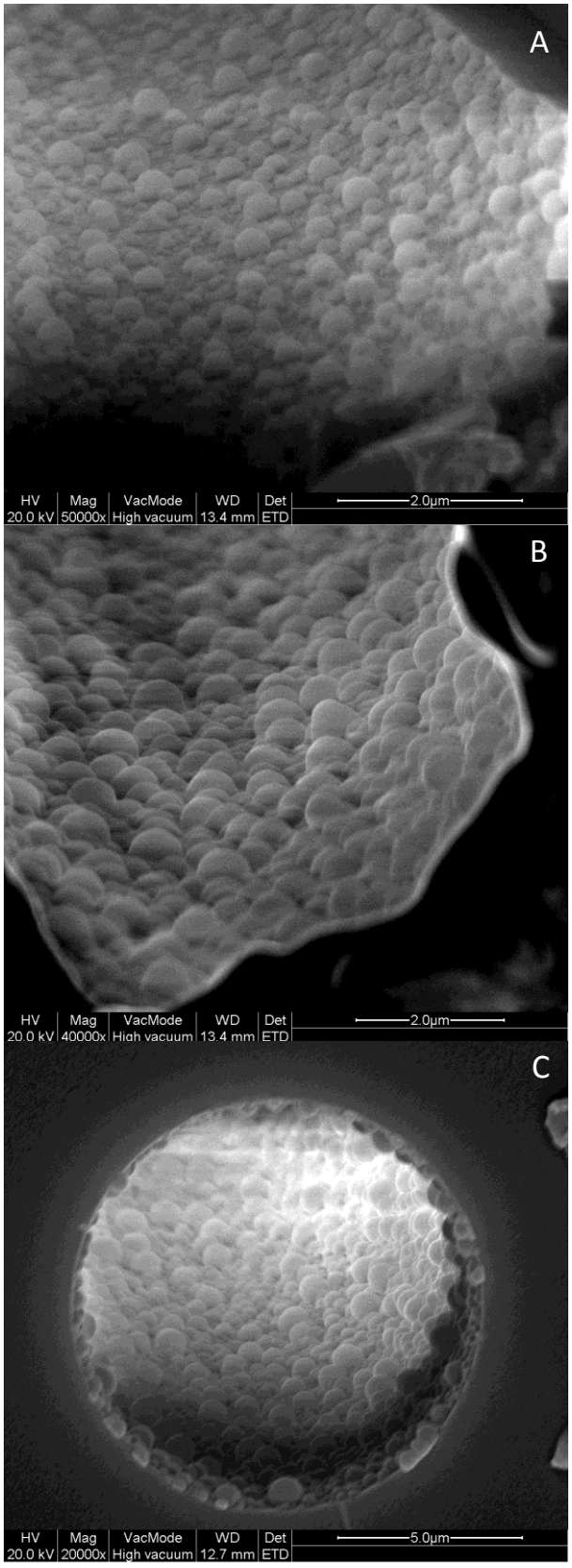
Table 1: The influence of the VSA ratio on the efficiency and retention factor. The monomer ratios are expressed as the relative v/v amounts of styrene, divinylbenzene and sulphonic acid, respectively.

Table 2

	Efficiency N (plates)			Retention factor (k or k_{app})			
Compound	25°C	40°C	60°C	25°C	40°C	60°C	Grad
methylparaben	73245	85577	64505	0.16	0.13	0.09	0.14
ethylparaben	53622	65066	46848	0.33	0.26	0.18	0.22
propylparaben	24364	39724	26816	0.5	0.39	0.28	0.30
butylparaben	8790	17586	14897	1.0	0.83	0.67	0.69

Table 2: The efficiency and retention factors of the parabens, analyzed at different set temperatures and thermal gradients.

Figure 1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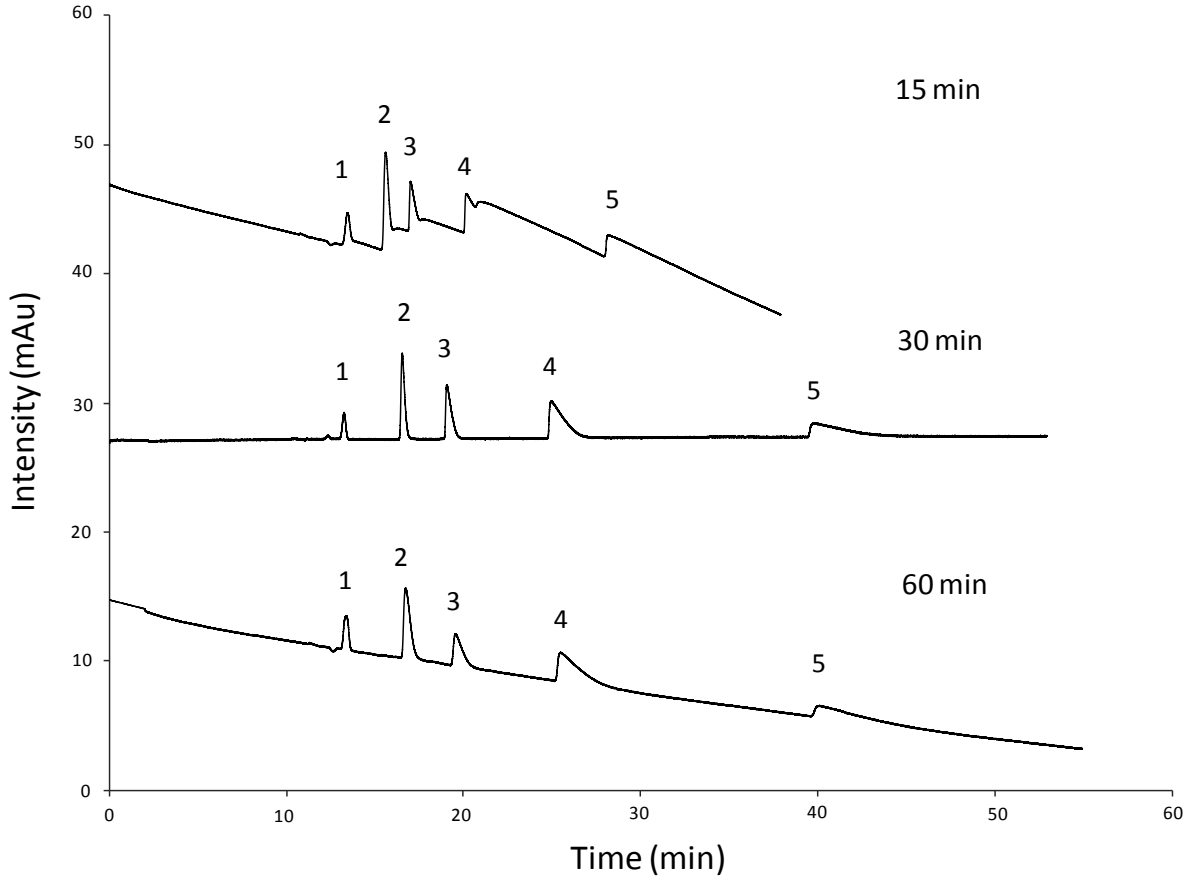
HV
20.0 kV
Mag
50000x
VacMode
High vacuum
WD
13.4 mm
Det
ETD
2.0µm
B
HV
20.0 kV
Mag
40000x
VacMode
High vacuum
WD
13.4 mm
Det
ETD
2.0µm
C
HV
20.0 kV
Mag
20000x
VacMode
High vacuum
WD
12.7 mm
Det
ETD
5.0µm

Figure 2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Time (min)
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30 min
60 min
1
2
3
4
5
0
10
20
30
40
50
60

Figuur 3

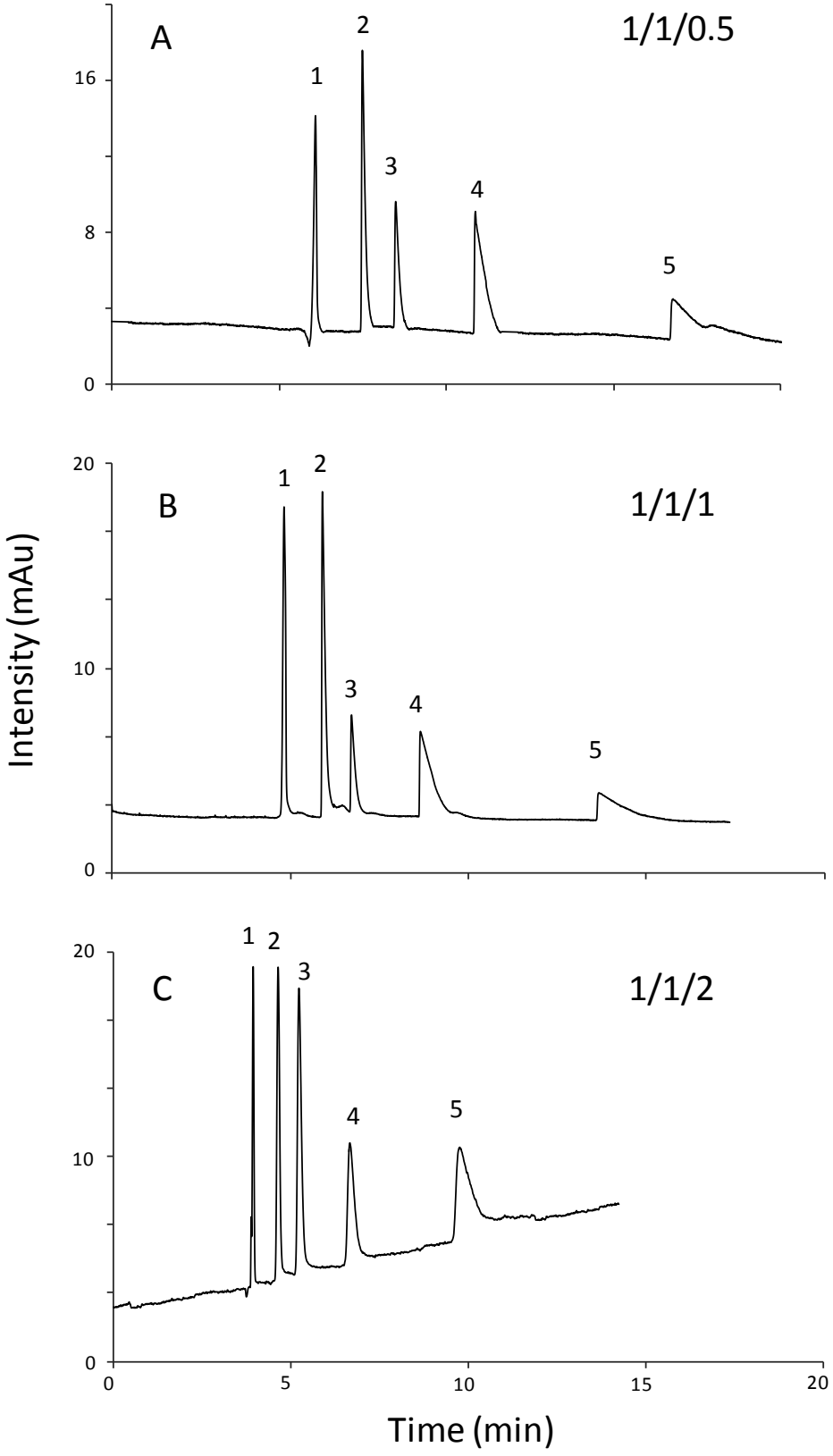
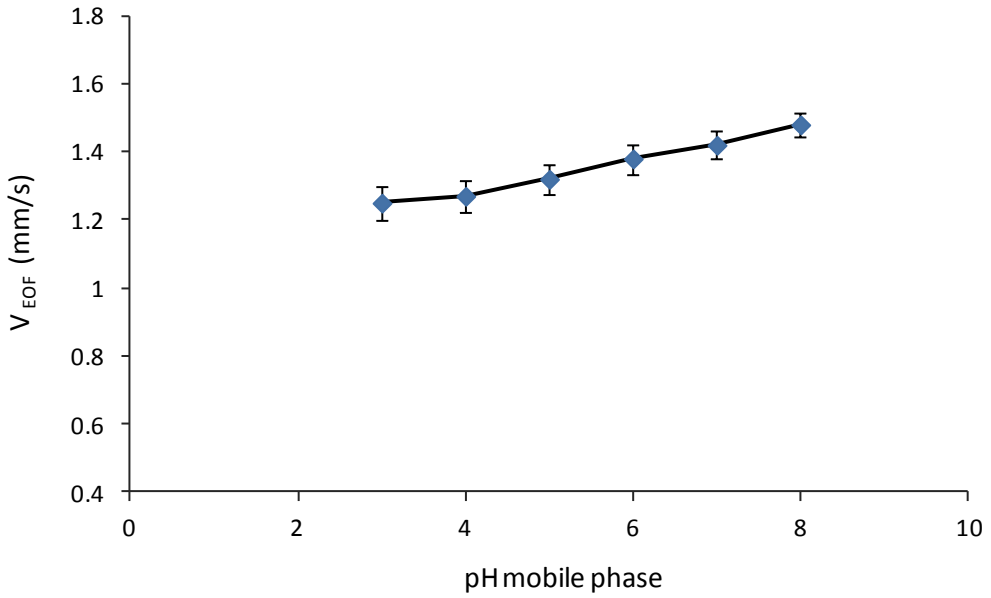
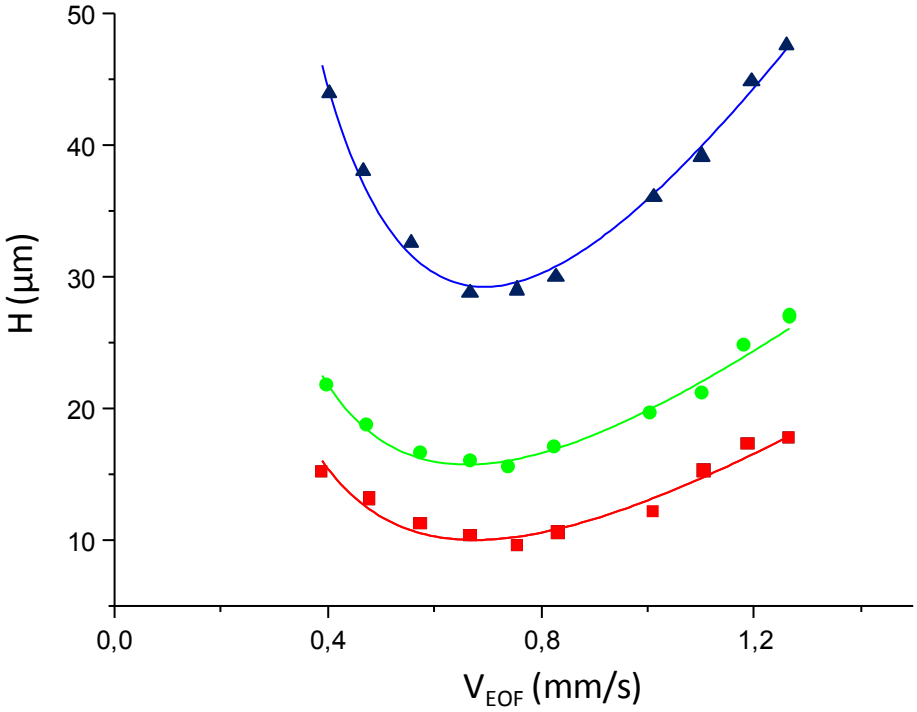

A
1/1/0.5
1
2
3
4
5
16
8
0
B
1/1/1
20
10
0
C
1/1/2
Intensity (mAu)
0
5
10
15
20
Time (min)

Figure 4



Figuur 5



Figuur 6

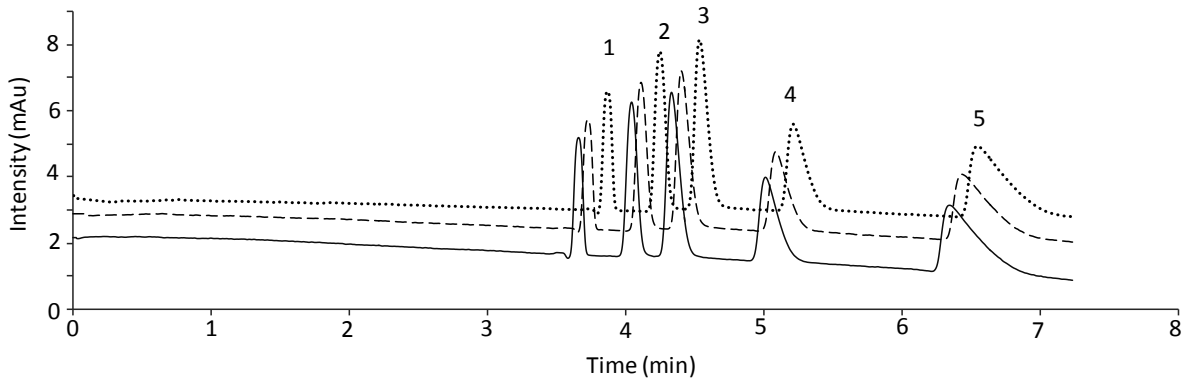


Figure 7